AN AUTORADIOGRAPHIC INVESTIGATION OF COELOMOCYTE PRODUCTION IN THE PURPLE SEA URCHIN (STRONGYLOCENTROUS PURPURATUS) [1]

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Coelomocyte is a generic term for several cell types which circulate in the fluid of the coelomic cavities and water vascular system of sea urchins. Boolootian and Giese (1958) have described the living coelomocytes from the perivisceral coelom of *Strongylocentrotus purpuratus*. They recognized the following seven cell types: bladder amebocytes, filiform amebocytes, colorless spherule amebocytes, eleocytes, vibratile cells, fusiform corpuscles, and hyaline hemocytes. It is well known that two types of coelomocyte, the eleocytes and colorless spherule amebocytes, not only circulate in the coelomic and water vascular fluid, but also wander freely throughout most tissues and organs of the urchin. Several workers have claimed that bladder and filiform amebocytes may occur in the gut wall (Kindred, 1926; Saint-Hilaire, 1897). What little is known of the functions of echinoid coelomocytes was summarized by Boolootian and Giese (1958, 1959).

Echinoid coelomocyte renewal has long been a subject of speculation and controversy. Several organs and tissues have been named, chiefly on histological evidence, as the site of coelomocyte production in sea urchins. A number of workers believed that the axial organ was the site of coelomocyte production (see Kollmann, 1908, p. 184). Kollmann claimed that, in addition to the cells of the axial organ, some types of circulating coelomocytes divided to produce more coelomocytes. Kollmann explained his inability to find any mitotic activity in the circulating coelomocytes or axial organ cells by implying that coelomocyte production was a discontinuous process, and coelomocytes were not being produced in the urchins at the time he investigated them. Like Kollmann, other workers have remarked on the rarity or complete absence of mitosis in the circulating coelomocytes of urchins. Geddes (1880) reported that he had only once or twice seen circulating coelomocytes divide. Yeager and Tauber (1935) looked in vain for mitotic figures in living coelomocytes from 20 specimens of *Arbacia punctulata*. Schinke (1950) was unable to find a single mitotic figure in living coelomocytes from 100 specimens of *Psammechinus miliaris*. Schinke also failed to detect any mitotic figures in fixed and stained coelomocytes. In the same paper, Schinke reported that the dermal connective tissue cells of the body wall were the source of at least some types of coelomocyte. Finally, the epithelial cells of the peritoneum have been named as the source for some or all coelomocyte types (Geddes, 1880; Sainte-Hilaire, 1897; Liebman, 1950).

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There have been several reports of the differentiation of one type of coelomocyte into another type in sea urchins. The term differentiation is used here to exclude the pre-clot transformation of the bladder amebocytes into filiform amebocytes, which is a short-term physiological change (Boolootian and Giese, 1959). Frenzel (1892) believed that eleocytes differentiated into bladder and filiform amebocytes. Kindred (1926) reported that bladder and filiform amebocytes were transformed into colorless spherule amebocytes, which were subsequently converted to eleocytes. Liebman (1950) held that the vibratile cells, after originating from the peritoneum, transformed into bladder and filiform amebocytes. Liebman further proposed a possible interconversion between eleocytes and colorless spherule amebocytes. Schinke (1950) reported that the colorless spherule amebocytes, after arising from connective tissue cells of the dermis, possibly differentiated into bladder and filiform amebocytes.

The diversity of opinions concerning the origin and interrelationships of echinoid coelomocyte types prompted the present autoradiographic investigation employing tritiated thymidine. Autoradiograms were prepared from tissues of sea urchins killed one hour after injection of tritiated thymidine, to determine the possible sites of coelomocyte (or coelomocyte precursor) proliferation. Autoradiograms were prepared from coelomocytes withdrawn from urchins at increasing time intervals after injection to demonstrate the possible transformation of labeled precursor cells into initially-unlabeled coelomocyte types. The circulating coelomocytes and the cells of the parietal peritoneum were studied quantitatively, since they could be prepared without histological sectioning, and yielded more reliable tritium indices than cells in sections. Autoradiograms of tissues that required sectioning were studied qualitatively. Coelomocyte production was investigated in starved as well as in freshly collected urchins.

Materials and Methods

All specimens of *Strongylocentrotus purpuratus* investigated were collected from tide pools near Yankee Point, California, and were sexually mature. The weight and sex of each freshly collected urchin are given in Table I. On the day of collection, these urchins were injected intracoelomically *via* the peristomial membrane with 0.2 μc. of tritiated thymidine per gram of fresh weight. One part of the aqueous solution of methyl-tritiated thymidine (obtained from New England Nuclear Corp., Boston, and having a specific activity of 6700 mc. per m*M*) was diluted with four parts of sea water before injection. The injected urchins were returned to containers of sea water for 50 minutes. Then coverslip preparations of their coelomocytes were made by the following method:

A 10% (w/v) aqueous solution of disodium ethylene-diaminetetraacetic acid (EDTA), adjusted to pH 8.1 by slow addition of solid NaOH, was used as an anticoagulant. A 1.0-ml. syringe (fitted with a 0.5-inch, 27-gauge needle) was filled with 0.2 ml. of the EDTA anticoagulant. The needle was introduced into the perivisceral coelom of an urchin by puncturing the peristomial membrane in a radial position, and a 0.2 ml.-sample of coelomic fluid was withdrawn. The contents of the syringe were then quickly ejected into a flat-bottomed shell vial (15 ml. capacity, inside diameter 22 mm., and height 5 mm.) containing 5 ml. of the EDTA anticoagulant and a circular coverslip 18 mm. in diameter. The

shell vial was placed in a regular centrifuge head, and the coelomocytes were spun down onto the coverslip at 500 rpm for ten minutes. The coverslip was then removed from the vial, and the adhering coelomocytes were fixed in formalin vapor under a bell jar. After overnight fixation, the still-moist coverslip preparation was placed in two successive 10-minute baths of distilled water. The preparation was subsequently placed in a bath of 5% acetic acid (aqueous) for 30 minutes to remove all traces of acid-soluble tritiated material adsorbed to the slide. The coverslip was rinsed in distilled water, dried, and finally glued, cell-side-up, to a microscope slide with Permount.

TABLE 1

Tritium indices of coelomocytes and peritoneal cells from urchins killed one hour after injection

Urchin weight	Sex	Blad.-fil. amebocyte		Col. sph. amebocyte		Eleocyte		Vibratile cell		Peritoneal cell	
		Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell
17.5 g.	M	3.3%	32	0.2%	18	1.4%	27	0.0%	—	1.6%	22
15.1 g.	F	5.5%	40	1.1%	30	3.6%	37	0.0%	—		
16.5 g.	M	1.5%	40	0.7%	19	2.6%	34	0.0%	—	1.2%	26
16.0 g.	F	2.5%	18	1.1%	5	1.9%	13	0.0%	—	1.8%	17
17.0 g.	M	1.0%	24	1.0%	19	1.4%	21	0.0%	—	0.4%	15
16.8 g.	F	1.6%	27	0.5%	12	1.2%	15	0.0%	—	0.6%	27
16.0 g.	M	0.9%	36	0.3%	16	0.2%	32	0.0%	—	1.1%	17
18.1 g.	M	0.5%	31	0.6%	11	0.4%	30	0.0%	—	0.5%	33
18.1 g.	F	3.7%	37	2.8%	17	5.1%	38	0.0%	—	1.3%	27
27.2 g.	M	3.0%	38	0.3%	14	2.7%	26	0.0%	—		
35.7 g.	F	3.7%	31	0.0%	—	3.7%	34	0.0%	—		
29.4 g.	F	1.7%	24	0.0%	—	1.4%	27	0.0%	—		
Average tritium index + standard deviation		2.4 ± 1.5%		0.7 ± 0.8%		2.1 ± 1.6%		0.0 ± 0.0%		1.1 ± 0.5%	

After the coelomocyte sample had been taken, each urchin was replaced in its container for 10 more minutes and then fixed in sea water-Bouin's fluid. The Bouin's fluid had completely decalcified the urchins within one week. After decalcification, the urchins were washed in water and dehydrated as far as 70% ethanol. At this point, samples of the following organs were dissected out for histological sectioning at 20 μ, 7 μ and 2 μ: gonad, axial organ, pharynx, esophagus, stomach, intestine, Polian vesicle with tooth, interradial body wall and radial body wall with radial nerve and water vascular elements attached. At this time, the parietal peritoneum with its supporting basement membrane was stripped off one interradial sector of body wall simply by seizing it with forceps and pulling. After a rinse in distilled water, the stripped peritoneum was placed on a microscope slide and teased with two pins until it was spread out in a flat sheet with

no wrinkles. The sheet was mounted with the basement membrane facing the surface of the slide and the coelom-bordering epithelial cells up. Mesenteric strands (anchoring the gut to the body wall in intact urchins) arose from the coelomic side of the peritoneum and aided in its orientation. Once the peritoneum had dried, it became firmly attached to the slide.

The stripped preparations of parietal peritoneum, the coverslip preparations of coelomocytes and the sectioned tissues were covered with Kodak AR-10 autoradiographic stripping film. The peritoneum and coelomocyte preparations were exposed for three weeks at 4° C. The sectioned tissues were exposed for 8 and

TABLE II

Tritium indices of coelomocytes and peritoneal cells from starved urchins killed one hour after injection

Aug. 27 Urchin weight	Sex	Blad.-fil. amebocyte		Col. sph. amebocyte		Eleocyte		Vibratile cell		Peritoneal cell	
		Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell	Tritium index	Av. # grains per labeled cell
14.7 g.	M									0.1%	50
33.9 g.	M									0.3%	33
24.1 g.	F	0.3%	50	0.0%	—	0.0%	—	0.0%	—	0.2%	50
28.7 g.	M	0.5%	50	0.0%	—	0.0%	—	0.0%	—	0.1%	33
33.9 g.	M	0.7%	50	0.0%	—	0.0%	—	0.0%	—	0.1%	20
19.2 g.	M	0.2%	40	0.0%	—	0.0%	—	0.0%	—	0.2%	31
21.4 g.	M	1.0%	50	0.7%	50	0.1%	50	0.0%	—	1.1%	36
34.3 g.	F	0.9%	34	0.5%	17	0.5%	34	0.0%	—	0.3%	30
22.8 g.	F	1.3%	50	0.0%	—	0.0%	—	0.0%	—		
22.3 g.	M	1.6%	50	0.0%	—	0.0%	—	0.0%	—		
Average tritium index ± standard deviation		0.8 ± 0.5%		0.2 ± 0.3%		0.1 ± 0.2%		0.0 ± 0.0%		0.3 ± 0.3%	

21 weeks at 4° C. After photographic processing (six minutes in Kodak D-19 developer at 18° C.), the coelomocytes and peritoneal cells were stained through the film with a 0.1% aqueous solution of toluidine blue for one minute. The emulsion was then destained for several minutes in 70% ethanol. After a rinse in tap water, the stained autoradiograms were dried, and immersion oil was placed directly on the film to observe them. For quantification, a labeled cell was defined as a cell having four or more silver grains above its nucleus. For each urchin, a minimum of 1000 cells was counted for each cell type quantified. For each cell type, the *tritium index* equalled $\frac{\text{number of labeled cells}}{\text{total number of cells counted}} \times 100$. The average number of grains per labeled cell was recorded with each tritium index. The autoradiograms of the sectioned

tissues were stained through the emulsion with toluidine blue or with nuclear fast red by the procedure of Montruil-Langlois (1962), omitting the counterstain.

Circulating coelomocytes from three uninjected donor urchins were exposed to tritiated thymidine *in vitro*. Each 30-g. donor was treated in the following way: By peristomial puncture, 0.2 ml. of perivisceral coelomic fluid was taken into a syringe containing 0.2 ml. of the EDTA anticoagulant at 14° C. The contents of the syringe was then ejected into a shell vial containing a circular coverslip and 5 ml. of the EDTA anticoagulant at 14° C. One μc. of tritiated thymidine in 0.01 ml. of distilled water was then added to the shell vial, and its contents were kept at 14° C. for 20 minutes. At least some of the cells collected in this way and subjected to washing with the EDTA anticoagulant are capable

TABLE III

Tritium indices of coelomocytes from three urchins serially sampled following a single injection of tritiated thymidine: the average number of grains per labeled cell is given in parentheses

		Interval from injection to samp ing			
		1 hour	24 hours	29 days	57 days
Sea urchin A	Blad.-fil. amebocyte	3.0% (38)		5.2% (21)	4.6% (17)
	Col. sph. amebocyte	0.3% (14)		4.9% (13)	7.9% (13)
	Eleocyte	2.7% (26)		4.8% (19)	5.9% (21)
	Vibratile cell	0.0% (—)		1.2% (17)	2.7% (16)
Sea urchin B	Blad.-fil. amebocyte	3.7% (31)	3.9% (31)		5.5% (19)
	Col. sph. amebocyte	0.0% (—)	0.1% (5)		5.0% (13)
	Eleocyte	3.7% (34)	1.9% (32)		6.9% (22)
	Vibratile cell	0.0% (—)	0.0% (—)		0.7% (15)
Sea urchin C	Blad.-fil. amebocyte	1.7% (24)	5.9% (31)	6.0% (26)	
	Col. sph. amebocyte	0.0% (—)	1.0% (23)	6.3% (11)	
	Eleocyte	1.4% (27)	5.7% (28)	8.8 % (21)	
	Vibratile cell	0.0% (—)	0.0% (—)	0.3% (20)	

of proliferation in tissue culture (Phillips and Farmanfarmaian, unpublished data). Therefore, exposure to the EDTA anticoagulant does not appear to affect the viability of the cells. Autoradiograms of the *in vitro*-labeled coelomocytes were prepared by the methods already described, the only difference being that they were exposed for eleven days rather than three weeks.

Each of ten urchins, which had been starved in the laboratory from April 19 to August 27, 1963, was injected with tritiated thymidine on August 27 and killed one hour later. The weight and sex of each starved urchin are given in Table II. The urchins were injected and sampled by the procedures already described for the freshly collected urchins. Coelomocytes, peritoneal cells, and other tissues were prepared for autoradiography by the procedures already given. The only difference was that the amount of perivisceral coelomic fluid withdrawn was increased from 0.2 ml. to 0.8 ml., since the concentration of coelomocytes in the coelomic fluid was lowered by the 130-day starvation.

The perivisceral coelomic fluid from three urchins (A, B and C of Table III) was serially sampled following a single injection of tritiated thymidine. The urchins were injected and sampled by the procedures already described for the freshly collected urchins. From each urchin, a 0.2-ml. sample of coelomic fluid was withdrawn one hour, 24 hours, 29 days and 57 days after the initial injection of tritiated thymidine. Autoradiograms were then made from the coverslip preparations of coelomocytes. Throughout the experiment, the three urchins were fed continuously on the alga, *Macrocystis*. Each of the 30-g. urchins gained about 5 g. during the 59 days of the experiment. After the final sampling of coelomocytes, the three urchins were killed, and their peritoneal cells and other tissues were prepared for autoradiography by the procedures already described.

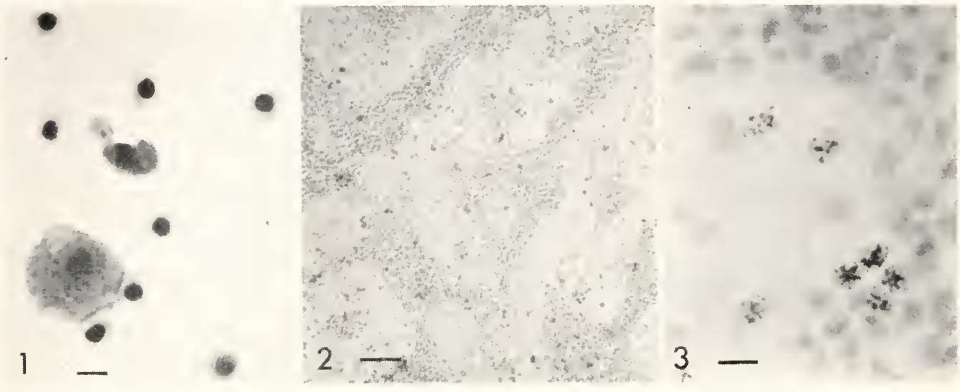

FIGURE 1. A toluidine blue-stained coverslip preparation of coelomocytes from the perivisceral coelom of *S. purpuratus*. In the field are seven despherulated eleocytes, one colorless spherule amebocyte (with a cytoplasmic process extending toward the upper left), one swollen vibratile cell and one bladder-filiform amebocyte (at lower right). The scale line is 5 μ long.

FIGURE 2. A stripped preparation of interradial parietal peritoneum showing the regions of sparsely distributed nuclei and the regions of closely packed cells in surface view. Toluidine blue. The scale line is 30 μ long.

FIGURE 3. A toluidine blue-stained autoradiogram of a stripped preparation of the parietal peritoneum from an urchin killed 57 days after injection of tritiated thymidine. The plane of the silver grains is in focus. The scale line is 5 μ long.

It was important to correlate the fixed and stained coelomocyte types, seen in coverslip preparations, with the living coelomocyte types described by Boolootian and Giese (1958). To accomplish this, a cross was scratched on one side of a circular coverslip, and coelomocytes were spun down onto the cross-bearing surface by the method already described. Prior to fixation, the living coelomocytes in the field with the cross were photographed. After fixation, the coelomocytes in the field with the cross were relocated and photographed a second time. The preparation was then stained in toluidine blue, and the same coelomocytes were photographed a third time.

RESULTS

The photographic correlation of the living coelomocyte types with the fixed and stained coelomocytes demonstrated that the vibratile cells, eleocytes, and

colorless spherule amebocytes could be reliably identified in autoradiograms. The vibratile cells swell when fixed and flatten into discs 6 μ to 20 μ in diameter when dried onto the slide. Toluidine blue stains the vibratile cell nuclei light blue and the cytoplasm (which contains an acid mucopolysaccharide) violet to purple. The flagellum is rarely visible in stained vibratile cells (Fig. 1).

Colorless spherule amebocytes retain their spherules when fixed (Fig. 1). Toluidine blue stains their nuclei dark blue and their spherules light green. When fixed in formalin vapor, most but not all of the circulating eleocytes lose their spherules. Eleocytes without spherules typically have round nuclei 4 μ in diameter which stain dark blue with toluidine blue; their cytoplasm does not stain (Fig. 1). The eleocytes which retain their spherules are often swollen to the size of the vibratile cell in Figure 1, and toluidine blue staining reveals large blue spherules (up to 3 μ in diameter) in their cytoplasm. It is probable that the circulating eleocytes which retain their spherules when fixed are identical to the orthochromatic coelomocytes described wandering in the gut wall of *S. purpuratus* (Holland and Nimitz, 1964). The bladder amebocytes, filiform amebocytes, fusiform corpuscles and hyaline hemocytes of Boolootian and Giese (1958) could not be reliably distinguished from one another when fixed and stained. For the purpose of this investigation, these four cell types are considered to be a single type, the bladder-filiform amebocyte (Fig. 1). The fixed and stained bladder-filiform amebocytes are variable in shape and size (5 μ to 10 μ in diameter exclusive of cytoplasmic processes). They typically have a lightly staining nucleus and a basophilic cytoplasm which is often, but not always, extended into processes.

The data gathered from autoradiograms of circulating coelomocytes withdrawn from freshly collected urchins 50 minutes after injection of tritiated thymidine are presented in Table I. The circulating bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes synthesize DNA, while the circulating vibratile cells do not. The average tritium indices for the circulating bladder-filiform amebocytes, colorless spherule amebocytes, eleocytes and vibratile cells labeled *in vivo* are 2.4%, 0.7%, 2.1%, and 0.0%, respectively. Average tritium indices for the bladder-filiform amebocytes, colorless spherule amebocytes, eleocytes and vibratile cells removed from three urchins and labeled *in vitro* are 2.8%, 0.2%, 1.5% and 0.0%, respectively. Thus, the circulating coelomocytes labeled *in vitro* immediately after removal from the urchin have tritium indices similar to those of Table I. These results rule out the possibility that all the labeled coelomocytes of Table I incorporated tritiated thymidine outside the coelomic circulation as they were finishing DNA synthesis and then entered the coelomic fluid in the 50 minutes between injection and sampling.

Table I shows a striking variability in coelomocyte tritium indices between individual urchins. Since all urchins listed were treated at approximately the same time of day, the variability is not due to a diurnal cycle of fluctuating tritium indices. Such fluctuations in tritium indices do occur in some cell types in mice (Pilgrim *et al.*, 1963). It also appears from Table I that, within the coelomic fluid of each individual, the tritium indices for the bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes are correlated—when one is high, the other two tend to be high also. To test the validity of this impression,

a correlation coefficient analysis was performed according to instructions in Moroney (1958, pp. 336–340). The analysis, the details of which are given in Holland (1964), demonstrates that the correlation is significant at the 5% level. This significant correlation suggests that DNA synthesis in the circulating bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes is under the control of one or more common factors present in the plasma of the coelomic fluid. Possible controlling factors might include either hormonal substances or nutrients necessary for DNA synthesis.

Presumably, DNA synthesis is eventually followed by mitosis of the circulating coelomocytes. In autoradiograms of coelomocytes withdrawn from urchins 24 hours after injection of tritiated thymidine, a number of labeled coelomocytes have two groups of silver grains above them, indicating that anaphase or telophase is occurring in the cell beneath. However, no chromosomes can be seen beneath the grains. Unequivocal mitotic figures also can not be detected in coverslip preparations of coelomocytes fixed in sea water-Bouin's fluid or Carnoy's acetic alcohol and stained with hematoxylin. It is apparent that some, and perhaps all, of the circulating coelomocytes which synthesize DNA subsequently divide in the coelomic fluid, but the mitotic figures are very difficult (if not impossible) to detect by ordinary histological methods. This undoubtedly explains the failure of earlier workers to detect mitosis in sea urchin coelomocytes (Kollmann, 1908; Yeager and Tauber, 1935; Schinke, 1950). It is interesting to note that Phillips and Farmanfarmaian (unpublished data) found that coelomocytes taken from the perivisceral coelom of *S. purpuratus* proliferated when cultured for several weeks *in vitro*. When coverslip cultures were fixed in Kahle's alcoholic formolacetic acid and stained with hematoxylin, some of the cultured coelomocytes showed all stages of mitosis with distinct chromosomes.

In addition to coelomocyte tritium indices, Table I presents tritium indices for the cells of the parietal peritoneum labeled for one hour *in vivo*. The interradial parietal peritoneum (Fig. 2) consists of regions of closely packed cells with elongate, often reniform, nuclei and regions of sparsely distributed, oval nuclei; cells of both regions are flagellated. The regions of closely packed cells run in interconnecting tracts, which may be seen in living, as well as fixed, stripped preparations (living peritoneum may be stripped from the dermis in small pieces). A one hour's exposure to tritiated thymidine labels some cells in both regions. For calculating the tritium index, cells of both regions were counted together. Some, and presumably all, of the peritoneal cells which synthesize DNA subsequently divide, since autoradiograms of peritoneum from urchins killed 57 days after injection of tritiated thymidine show that labeled cells (in both regions) frequently occur in groups of two to four (Fig. 3). Groups of more than four labeled cells also occur, but are less easily identified as progeny of a single initially-labeled cell.

In addition to cells of the parietal peritoneum, cells of all other tissues named by earlier workers as sites of coelomocyte production synthesize DNA. Autoradiograms of sectioned tissues from freshly collected urchins killed one hour after injection show some labeled cells in the radial parietal peritoneum, in the visceral peritoneum, in the epithelium lining all parts of the water vascular system, in the axial organ, in the Polian vesicles, in the hemal strands and in the dermal

connective tissues of the body wall. Furthermore, there is DNA synthesis by some of the eleocytes and colorless spherule amebocytes wandering in the body wall and gut wall. Cells of other tissues also synthesize DNA, but are not listed here since they have not been implicated in coelomocyte production. All tissues named as sites of coelomocyte production still contain labeled cells in urchins killed 57 days after injection.

The tritium indices of coelomocytes and peritoneal cells from the starved urchins killed one hour after injection are presented in Table II. The average tritium indices of bladder-filiform amebocytes, eleocytes and peritoneal cells are significantly lower in starved than in freshly collected urchins. Due to large standard deviations, the average tritium index of colorless spherule amebocytes is not significantly lower (at the 5% level) in starved than in freshly collected urchins. The circulating vibratile cells, in the starved as in the freshly collected urchins, never synthesize DNA. Autoradiograms of sectioned tissues from the starved urchins showed that a few cells of all regions implicated in coelomocyte production synthesize DNA. The average number of grains per labeled cell is higher in starved than in freshly collected urchins, perhaps because a smaller number of cells synthesizing DNA results in less competition for the injected tritiated thymidine, perhaps because of a lesser dilution of the tritiated thymidine by unlabeled endogenous thymidine.

After four months' starvation, protein in the perivisceral fluid of *S. purpuratus* declined from .37 mg. per 100 ml. to .14 mg. (L. Holland, unpublished). After 41 days' starvation, lipid in the perivisceral fluid declined from 25 mg. per 100 ml. to 15 mg. and carbohydrate from 2 mg. per 100 ml. to 1 mg. (A. Lawrence and J. Lawrence, unpublished).

The tritium indices for the coelomocytes of the serially sampled urchins are presented in Table III. In donors A and C, labeled vibratile cells appear in the circulation sometime between 24 hours and 29 days after injection of tritiated thymidine. The 29-day sample for donor B was not countable, but labeled vibratile cells had entered its circulation by 57 days after injection. Therefore, some proliferating cell type must be differentiating into vibratile cells. The time course for this differentiation is on the order of several days at the least and several weeks at the most. The serial results for the bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes are not very consistent. In most cases, the tritium index rises with time, but sometimes it drops. The striking rise in the tritium index of all three coelomocyte types of urchin C between one hour and 24 hours is unexpected and will be discussed below. The average number of grains per labeled cell usually falls slowly with time but in several instances remains constant or rises.

Discussion

The circulating vibratile cells of *S. purpuratus* are mature, non-proliferating cells, which must necessarily differentiate from one or more proliferating cell populations somewhere in the sea urchin. Of the many possible sources of vibratile cells, the cells of the parietal peritoneum are the most likely. The cytoplasm of some cells of the parietal peritoneum is filled with granules which stain violet with toluidine blue (*i.e.*, show B-metachromasia) and which may be a mucopolysac-

charide similar to the one found in mature vibratile cells. Such a mucopolysaccharide does not occur in the other cell populations which have been implicated in coelomocyte production. Furthermore, as Liebman (1950) pointed out, each cell of the peritoneum bears a single flagellum, which could be a precursor of the longer flagellum of a vibratile cell. Since the vibratile cell is a non-dividing, differentiated cell type, it is most unlikely that it transforms into other coelomocyte types as Liebman claimed in 1950.

The lack of DNA synthesis in circulating vibratile cells, as well as the appearance of labeled vibratile cells in the circulation in the weeks following injection, does not support the suggestion of Cuénot (1948, p. 149) that vibratile cells are not urchin cells at all, but are parasitic flagellate protozoans of the genus *Oikomonas*. The conclusion that the vibratile cells are sea urchin cells and not parasites is further supported by Boolootian's 1962 report that the perivisceral fluid in all individuals of twelve different species of regular echinoids contained vibratile cells. Such universal distribution and lack of specificity is not characteristic of a parasite.

Some of the bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes of *S. purpuratus* synthesize DNA and apparently divide. All new coelomocytes could arise by division of pre-existing coelomocytes, which would thus constitute self-maintaining cell populations. Cuénot (1897, p. 185) proposed just such a system for replacement of circulating cells of some arthropods, some annelids and some molluscs. However, all circulating and tissue coelomocytes synthesizing DNA could go through only one or a few growth-duplication cycles and thus constitute an amplification compartment of a cell system which must necessarily have a self-maintaining compartment somewhere in the urchin. This self-maintaining, ultimate source of coelomocytes could be one or more of the tissues which have been named as sites of coelomocyte production, since cell proliferation occurs in all of them. There are many possible schemes between these extremes. For example, only one of the three coelomocyte types could be a self-maintaining cell population. Furthermore, tissue coelomocytes might have a different renewal scheme from circulating coelomocytes of the same type. There is also the possibility (which was neither proved nor disproved in the present investigation) that one type of coelomocyte may differentiate into other types. The present lack of information on these points makes an unambiguous interpretation of Table III impossible.

Interpretation of the data in Table III is further hampered by the lack of information on the possible inter-changes of circulating and tissue coelomocytes and on the related question of whether or not the circulating coelomocytes are in a steady-state. It is known that large numbers of coelomocytes enter and leave the coelomic circulation in stressed urchins which have had all or part of their coelomic fluid removed (Schinke, 1950; Boolootian and Lasker, 1964). In the present investigation, urchins A, B, and C of Table III were probably stressed to some extent by withdrawal of 0.2 ml. (5% to 10%) of their coelomic fluid at each sampling. The sampling of urchin C one hour after injection could well have induced an immigration of tissue coelomocytes (having high tritium indices) into the coelom between one and 24 hours after injection.

In adult mammals, a renewing cell population has an initially-high tritium index; with time, the tritium index and the average number of grains per labeled

cell fall rapidly (Messier and Leblond, 1960). An expanding cell population in mammals has an initially-low tritium index; with time, the tritium index and the average number of grains per labeled cell tend to persist. The data in Table III demonstrate that sea urchin coelomocytes resemble expanding, rather than renewing cell populations, of mammals. Indeed, most of the cell populations of adult sea urchins have the characteristics of expanding cell populations. In the adult urchin, only the gametogenic cells of the male during the ripe season and the tooth cells have the characteristics of renewing cell populations (Holland, 1964).

The starved urchins of Table II showed no weight increase during starvation, although they appeared healthy in every other way. Presumably, the number of cells in their constituent cell populations was not increasing at the time of labeling on the last day of starvation. In most of the cell populations of the starved urchins, the few cells synthesizing DNA (33% of normal in the bladder-filiform amebocytes and 27% of normal in the peritoneal cells) are probably preparing to divide for replacement of cells lost from the populations and not for growth. Probably, even under normal conditions of growth, a minor component of proliferation in each expanding cell population is for replacement and not for growth. The reduction of tritium indices by starvation might be due to a reduction in the nutrients necessary for cell proliferation or to starvation-induced hormonal changes. Since it is unlikely that starvation shortens the DNA synthesis period, the lowering of the tritium indices is probably due to an increase in the duration of the growth-duplication cycle by a lengthening of the period of the interphase preceding the DNA synthesis period.

Summary

1. Brief exposure to tritiated thymidine *in vivo* or *in vitro* labels some of the bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes circulating in the perivisceral coelomic fluid of the purple sea urchin; circulating vibratile cells are not labeled. Some colorless spherule amebocytes and eleocytes are also labeled while wandering in the body wall and gut wall.

2. Some, and presumably all, of the circulating coelomocytes which synthesize DNA subsequently divide in the circulation. It is not known if this proliferation constitutes all or only a part of production of new bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes in the sea urchin.

3. The tritium indices of the circulating bladder-filiform amebocytes, colorless spherule amebocytes and eleocytes within each individual urchin are significantly correlated. This suggests that DNA synthesis in these circulating cells is under the control of one or more common factors.

4. Brief exposure to tritiated thymidine labels some cells in the visceral peritoneum, the parietal peritoneum, the epithelium lining all parts of the water vascular system, the axial organ, the Polian vesicles, the hemal strands and the dermal connective tissue of the body wall. These tissues have been named by previous authors as sites of coelomocyte production. The present investigation found no evidence for or against their participation in the production of bladder-filiform amebocytes, colorless spherule amebocytes or eleocytes.

5. In the weeks following injection of tritiated thymidine, labeled vibratile cells begin to appear in the coelomic fluid. The source of the vibratile cells is probably the parietal peritoneum. The vibratile cells thus constitute a sea urchin cell type and are not, as has been claimed, parasitic flagellates.

6. Serial autoradiographic data demonstrate that the coelomocytes (and most of the other tissues) of the young adult sea urchin have the characteristics of expanding, and not renewing, cell populations.

7. Starvation for several months causes a significant reduction in the tritium indices of the bladder-filiform amebocytes, eleocytes and cells of the parietal peritoneum.

LITERATURE CITED

Boolootian, R. A., 1962. The perivisceral elements of echinoderm body fluids. *Amer. Zool.*, **2**: 275–284.

Boolootian, R. A., and A. C. Giese, 1958. Coelomic corpuscles of echinoderms. *Biol. Bull.*, **115**: 53–63.

Boolootian, R. A., and A. C. Giese, 1959. Clotting of echinoderm coelomic fluid. *J. Exp. Zool.*, **140**: 207–229.

Boolootian, R. A., and R. Lasker, 1964. Digestion of brown algae and the distribution of nutrients in the purple sea urchin *Strongylocentrotus purpuratus*. *Comp. Biochem. Physiol.*, **11**: 273–289.

Cuénot, L., 1897. Les globules sanguins et les organes lymphoides des invertébrés. *Arch. Anat. Micr.*, **1**: 153–192.

Cuénot, L., 1948. Echinodermes, pp. 1–363. *In:* Traité de Zoologie, Vol. XI, ed. Grasse. Masson, Paris.

Frenzel, J., 1892. Beiträge zur vergleichende Physiologie und Histologie der Verdauung. 1. Mitheilung der Darmkanal der Echinodermen. *Arch. Anat. Physiol., Physiol. Abt.*, **16**: 81–114.

Geddes, P., 1880. Observations sur le fluide perivisceral des oursins. *Arch. Zool. Exp. Gen. (Ser. 1)*, **8**: 483–496.

Holland, N. D., 1964. Cell proliferation in post-embryonic specimens of the purple sea urchin (*Strongylocentrotus purpuratus*): an autoradiographic investigation employing tritiated thymidine. Doctoral Dissertation, Stanford University.

Holland, N. D., and S. A. Nimitz, 1964. An autoradiographic and histochemical investigation of the gut mucopolysaccharides of the purple sea urchin (*Strongylocentrotus purpuratus*). *Biol. Bull.*, **127**: 280–293.

Kindred, J. E., 1926. A study of the genetic relationships of the "amebocytes with spherules" in *Arbacia*. *Biol. Bull.*, **50**: 147–154.

Kollmann, M., 1908. Recherches sur les leucocytes et le tissue lymphoide des invertébrés. *Ann. Sci. Nat. Zool. (Sér. 9)*, **8**: 1–240.

Liebman, E., 1950. The leucocytes of *Arbacia punctulata*. *Biol. Bull.*, **98**: 46–59.

Messier, B., and C. P. Leblond, 1960. Cell proliferation and migration as revealed by radioautography after injection of thymidine H^3 into male rats and mice. *Amer. J. Anat.*, **106**: 247–265.

Montreuil-Langlois, M., 1962. Staining sections coated with radiographic emulsion: a nuclear fast red, indigo carmine sequence. *Stain Tech.*, **37**: 175–177.

Monroney, M. J., 1958. Facts from Figures. Penguin Books, Baltimore.

Pilgrim, C., W. Erb and W. Mauer, 1963. Diurnal fluctuations in the numbers of DNA synthesizing nuclei in various mouse tissues. *Nature*, **199**: 863.

Saint-Hilaire, C., 1897. Ueber die Wanderzellen in der Darmwand der Seeigel, Résumé. *Trav. Soc. Imp. Nat., St. Petersburg*, **27**(3): 221–248.

Schinke, H., 1950. Bildung und Ersatz der Zellelemente der Leibeshohlenflussigkeit von *Psammechinus miliaris* (Echinoidea). *Zeitschr. Zellforsch. mikr. Anat.*, **35**: 311–331.

Yeager, J., and O. Tauber, 1935. On the haemolymph cell counts of some marine invertebrates. *Biol. Bull.*, **69**: 66–70.